Vitamin D status and metabolism in an ovine pregnancy model: effect of long-term, high-altitude hypoxia

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Goyal R, Billings TL, Mansour T, Martin C, Baylink DJ, Longo LD, Pearce WJ, Mata-Greenwood E. Vitamin D status and metabolism in an ovine pregnancy model: effect of long-term, high-altitude hypoxia. Am J Physiol Endocrinol Metab 310: E1062–E1071, 2016. First published May 3, 2016; doi:10.1152/ajpendo.00494.2015.—Vitamin D status increases during healthy mammalian pregnancy, but the molecular determinants remain uncharacterized. The first objective of this study was to determine the effects of pregnancy, and the second objective was to examine the role of chronic hypoxia on vitamin D status and metabolism in an ovine model. We analyzed the plasma levels of cholecalciferol, 25-OH-D, and 1α,25-(OH)2-D in nonpregnant ewes, near-term pregnant ewes, and their fetuses exposed to normoxia (low altitude) or hypoxia (high-altitude) for 100 days. Hypoxic sheep had increased circulating levels of 25-OH-D and 1α,25-(OH)2-D compared with normoxic sheep. Hypoxia increases in 25-OH-D were associated with increased expression of renal 25-hydroxylases CYP2R1 and CYP2J. Pregnancy did not increase further the plasma levels of 25-OH-D, but it significantly increased those of the active metabolite, 1α,25-(OH)2-D, in both normoxic and hypoxic ewes. Increased bioactivation of vitamin D correlated with increased expression of the vitamin D-activating enzyme CYP27b1 and decreased expression of the inactivating enzyme CYP24a1 in maternal kidneys and placentas. Hypoxia increased parathyroid hormone levels and further increased renal CYP27b1. Pregnancy and hypoxia decreased the expression of vitamin D receptor (VDR) in maternal kidney and lung, with opposite effects on placental VDR. We conclude that ovine pregnancy is a model of increased vitamin D status, and long-term hypoxia further improves vitamin D status due to pregnancy- and hypoxia-specific regulation of VDR and metabolic enzymes.

Vitamin D status increases during healthy mammalian pregnancy, but the molecular determinants remain uncharacterized. The first objective of this study was to determine the effects of pregnancy, and the second objective was to examine the role of chronic hypoxia on vitamin D status and metabolism in an ovine model. We analyzed the plasma levels of cholecalciferol, 25-OH-D, and 1α,25-(OH)2-D in nonpregnant ewes, near-term pregnant ewes, and their fetuses exposed to normoxia (low altitude) or hypoxia (high-altitude) for 100 days. Hypoxic sheep had increased circulating levels of 25-OH-D and 1α,25-(OH)2-D compared with normoxic sheep. Hypoxia increases in 25-OH-D were associated with increased expression of renal 25-hydroxylases CYP2R1 and CYP2J. Pregnancy did not increase further the plasma levels of 25-OH-D, but it significantly increased those of the active metabolite, 1α,25-(OH)2-D, in both normoxic and hypoxic ewes. Increased bioactivation of vitamin D correlated with increased expression of the vitamin D-activating enzyme CYP27b1 and decreased expression of the inactivating enzyme CYP24a1 in maternal kidneys and placentas. Hypoxia increased parathyroid hormone levels and further increased renal CYP27b1. Pregnancy and hypoxia decreased the expression of vitamin D receptor (VDR) in maternal kidney and lung, with opposite effects on placental VDR. We conclude that ovine pregnancy is a model of increased vitamin D status, and long-term hypoxia further improves vitamin D status due to pregnancy- and hypoxia-specific regulation of VDR and metabolic enzymes.

VITAMIN D HAS WELL-ESTABLISHED CLASSIC EFFECTS on bone metabolism and mineral homeostasis (8, 33). In addition, vitamin D has important noncalcemic roles in the reproductive, cardiovascular, renal, lung, immune, neuronal, and pancreatic systems (8, 33). In reproduction, vitamin D deficiency has been associated with sterility, placental insufficiency, intrauterine growth restriction, recurrent miscarriages, gestational diabetes, and preeclampsia (maternal high blood pressure and proteinuria) (6, 51–53). Furthermore, maternal vitamin D deficiency has been also linked to postnatal infectious diseases, autoimmune diseases (diabetes type I), asthma, and obesity (26, 28, 54).

Vitamin D3 (cholecalciferol) can be produced endogenously from 7-dehydrocholesterol by sun exposure in the skin epidermis or obtained from dietary sources (8, 33). Vitamin D is activated endogenously by mitochondrial 25-hydroxylase enzymes that synthesize 25-OH-D, followed by a second hydroxylation at the 1α position that yields the active metabolite of vitamin D: 1α,25-(OH)2-D (calcitriol) (8, 33). This step is catalyzed by a rate-limiting enzyme, 25(OH)-1α-hydroxylase (CYP27b1), that is expressed abundantly in the renal cortex. In the blood, vitamin D metabolites are transported bound to albumin and the vitamin D-binding protein (VDBP) (8, 33). At the molecular level, vitamin D mediates its biological effects by binding and activating the vitamin D receptor (VDR) (14). Active VDR, heterodimerized with the retinoid X receptor, regulates gene expression by targeting gene promoters containing vitamin D response elements. This leads to activation or repression of transcription resulting in gene expression changes (14). A classic gene regulated in this manner is the vitamin D-inactivating enzyme CYP24A1 (24-hydroxylase). Most cells that express VDR also express CYP24A1, providing a unique negative feedback control mechanism to regulate the vitamin D effects (8, 14). In healthy nonpregnant mammals, the circulating levels of 1α,25-(OH)2-D are tightly regulated; low levels of 1α,25-(OH)2-D stimulate the release of parathyroid hormone (PTH) that upregulates renal CYP27b1 expression, and high levels of 1α,25-(OH)2-D upregulate VDR that upregulates CYP24A1 expression (8, 33).

The metabolism of vitamin D changes significantly during mammalian pregnancy (13, 40, 42). In humans and rats, maternal plasma levels of ionized calcium and the abundant precursor 25-(OH)-D do not change, but the circulating levels of active 1α,25-(OH)2-D increase severalfold from early pregnancy and remain high during the entire length of pregnancy (13, 40, 42). In human and rat placentas from healthy pregnancies the expression of CYP27b1 is increased, whereas the contrary is true for placental CYP24a1 expression, favoring the production of bioactive vitamin D (9, 10, 27, 38). However, the factors that regulate vitamin D metabolism during pregnancy remain incompletely characterized. For instance, CYP27b1 upregulation is not mediated by PTH that remains low during human and rodent pregnancy (18, 36). In addition, increases in vitamin D levels usually result in CYP24a1 upregulation in target organs such as kidney (20, 41), but this physiological event is blunted during normal pregnancy. Thus, the mechanisms that lead to higher plasma levels of 1α,25-(OH)2-D during normal pregnancy have not been fully elucidated and limit our ability to optimize vitamin D status (defined by the circulating levels of vitamin D metabolites) during pregnancy.

On a global basis, vitamin D deficiency during pregnancy is highly prevalent (17, 44, 50). Several developmental age,
Outcomes. Vitamin D status that correlate with adequate fetal and maternal adaptations are exposed to hypobaric hypoxia, a major risk factor for pregnancy complications. Epidemiological studies have revealed that high-altitude pregnancies are at higher risk of preeclampsia, intrauterine growth restriction, and perinatal mortality and morbidity (15, 16, 43, 50a). The susceptibility to high-altitude pregnancy and postnatal complications varies between mammal species; for instance, sheep are less sensitive than rats to hypoxia-induced pulmonary hypertension (39, 48). In humans, there is significant inter- and intrapopulation differences in response to high-altitude hypoxia, with native populations such as the Tibetans and the Andeans showing better pregnancy outcomes compared with their Chinese and European cohabitants (30). Although hypoxia and vitamin D deficiency are both risk factors for similar pregnancy complications, the individual roles of high-altitude hypoxia and vitamin D deficiency in mammalian pregnancy have not been elucidated.

Our center for Perinatal Biology has been investigating the maternal and fetal adaptations to high-altitude hypoxia, using a model in which the ewe is maintained at an altitude of 3,820 m from day 40 of gestation to near term (11, 31, 39). This model is characterized by decreases in both maternal and fetal arterial PO2 that lead to cardiovascular, pulmonary, neuronal, endocrine, and other systems adaptations. This is a model of acclimatization where the fetus is not growth restricted but shows increased blood pressure, decreased cardiovascular output, and altered vascular and neuroendocrine responses to secondary stressors (11, 31, 39). Understanding the regulation of vitamin D status in this model would further our understanding on the adaptations of this species to high-altitude hypoxia. Sheep, like humans and rodents, show an increase in maternal vitamin D levels during uncomplicated pregnancies (22); however, the molecular determinants of these changes remain uncharacterized. Recently, we have shown that healthy rodent pregnancies are characterized by maternal renal gene expression changes that include upregulation of CYP27b1 and downregulation of the VDR/CYP24a1 negative feedback loop (12). In this manner, pregnancy allows for significant increases in circulating levels of bioactive vitamin D without concomitant inactivation by CYP24a1. The first aim of this study was to uncover changes in vitamin D status and metabolism in pregnant sheep similar to those observed in pregnant rats. The second goal was to investigate a potential role of chronic hypoxia in vitamin D status and metabolism. We found that the control sea-level normoxic ewes have physiological changes in the vitamin D system similar to our healthy pregnant rats and that the high-altitude hypoxic ewes had further increases in vitamin D status that correlate with adequate fetal and maternal outcomes.

### Materials and Methods

**Animals and tissue collection.** The animal protocols for this study were submitted to, reviewed by, and approved by the Institutional Animal Care and Use Committee at Loma Linda University School of Medicine. All animals were fed alfafa pellets that are a standard nutritious diet for ruminants. Time-dated pregnant ewes (n = 7; 3 twin and 4 singleton) were maintained at the Barcroft Laboratory White Mountain Research Station (elevation 3,820 m) for ~110 days beginning on day 30 of gestation (term = 146 days). Age-matched nonpregnant ewes (n = 5) were also maintained at high altitude for similar length. The animals were then transported to Loma Linda University Medical Center Animal Research Facility (elevation 346 m). Normoxic, age-matched pregnant ewes (n = 7; 3 twin and 4 singleton) and nonpregnant ewes (n = 5) were used as controls. Between days 139 and 141 of gestation, ewes in each group were euthanized, and maternal and fetal umbilical cord blood was collected in heparinized tubes, and placenta, kidney cortex, liver, lung, and duodenum samples from both ewes and fetuses were snap-frozen in liquid nitrogen. Blood samples were processed to obtain plasma, followed by red blood cell lysis to obtain white blood cell fractions or peripheral blood mononuclear cells (PBMC).

**ELISA analysis of vitamin D metabolites, calcium, and VDBP plasma levels.** Plasma levels of 25-OH-D and 1α, 25-(OH)2-D were analyzed using commercially available ELISA kits that determine total (bound and free) levels of vitamin D metabolites (Immunodiagnostic Systems, Scottsdale, AZ). We have previously validated these ELISA kits by liquid chromatography-mass spectrometry/mass spectrometry analysis (12). Plasma levels of vitamin D (cholecalciferol) and vitamin D-binding protein (VDBP) were analyzed with the aid of commercially available ELISAs according to the manufacturer’s protocol (MyBiosource). Plasma levels of total calcium were assayed using a colorimetric kit (Abcam, San Francisco, CA), following the respective

### Table 1. Primers used for SYBR green real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Accession No.</th>
</tr>
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<tr>
<td>Bovine CYP27b1 Forward</td>
<td>5'-TGCTAAAGACTGTACCCTGTGG-3'</td>
<td>NM_001192284.1</td>
</tr>
<tr>
<td>Bovine CYP24a1 Forward</td>
<td>5'-CCGAAGAGGAGAAGTTGAA-3'</td>
<td>NM_001167932.1</td>
</tr>
<tr>
<td>Bovine CYP2R1 Forward</td>
<td>5'-GGTTCAGAGACCTCACCCCT-3'</td>
<td>NM_001076267.2</td>
</tr>
<tr>
<td>Bovine CYP2R1 Reverse</td>
<td>5'-ATGAGTCCTCGCTTCGTACCCG-3'</td>
<td>NM_001083413.2</td>
</tr>
<tr>
<td>Bovine CYP3a4 Forward</td>
<td>5'-TCGATCGAGTCCCTTTTCTTC-3'</td>
<td>NM_001099367.1</td>
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<td>Bovine CYP2J Forward</td>
<td>5'-GGTCCAGGTTGTTGTTGGAAGA-3'</td>
<td>NM_001077210.1</td>
</tr>
<tr>
<td>Bovine VDR Forward</td>
<td>5'-GACCCCGTCGCTGCTGGAAGT-3'</td>
<td>NM_001167932.1</td>
</tr>
<tr>
<td>Bovine VDR Reverse</td>
<td>5'-GGGTTGAGAAGAAGTGCTTGAG-3'</td>
<td>NM_001035380.2</td>
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<tr>
<td>Bovine megalin Forward</td>
<td>5'-AGAAAATCCTCCAAGCCCAAGCA-3'</td>
<td>AB863206.1</td>
</tr>
<tr>
<td>Bovine megalin Reverse</td>
<td>5'-AGAAAATCCTCCAAGCCCAAGCA-3'</td>
<td>NM_001192575.1</td>
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<tr>
<td>Bovine 18S Forward</td>
<td>5'-AACGCGGTACACATTCACAACG-3'</td>
<td>NY53190.1</td>
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<td>Bovine 18S Reverse</td>
<td>5'-TCCGTGATCTGTATTTTTGTTGCAC-3'</td>
<td>NM_001076267.2</td>
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VDR, vitamin D receptor; VDBP, vitamin D-binding protein.
manufacturer's instruction manuals. Normal plasma values reported for nonpregnant humans are 75–200 nM for 25-hydroxycholecalciferol and 25-OH-D, 50–195 pM for 1α,25(OH)2D, 300–550 mg/dl for VDBP, 8.5–11.8 mg/dl for total calcium, and 10–60 pg/ml for intact PTH (iPTH). Vitamin D status was defined by the plasma levels of all vitamin D metabolites, including cholecalciferol, 25-OH-D, and 1α, 25(OH)2D.

Quantitative real-time PCR. RNA isolation, RT reaction, and real-time PCR were performed as described previously (12). Exon spanning primers were obtained from the literature and confirmed by standardized efficiency testing. Samples were analyzed on the Roche LightCycler 1.5 (Roche, Indianapolis, IN) using the Quantitect SYBR green kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The designed primers and accession numbers are shown in Table 1. PCR products were confirmed by sequencing. Quantitative analysis was performed with the aid of standard curves using two different control plasmids, as described previously (12, 30).

Data are reported as fg mRNA divided by ng 18S RNA.

SDS-PAGE and immunoblotting. From each group, snap-frozen placentas, maternal and fetal kidney cortex, liver, duodenum, and lambs were studied for the circulating plasma levels of cholecalciferol (Chol), 25-hydroxy-D (25-OH-D), 1α,25(OH)2D, 25-OH-D, 1α,25(OH)2D, and 1α,25(OH)2D. SDS-PAGE and immunoblotting.

Fig. 1. Vitamin D status in an ovine model of pregnancy: effect of long-term hypoxia (LTH). Near-term pregnant (P) ewes, their fetuses (F), and age-matched nonpregnant (NP) ewes were studied for the circulating plasma levels of cholecalciferol (A), 25-OH-D (B), 1α,25(OH)2D (C), total calcium (D), the vitamin D-binding protein (VDBP; E), and intact parathyroid hormone (iPTH; F) as described in MATERIALS AND METHODS. Results are shown as averages ± SE (n = 5–7/group). *P < 0.05 LTH vs. normoxia (NMX); #P < 0.05 NP vs. P; †P < 0.05 F vs. P.

Methylated DNA immunoprecipitation. To determine the levels of vitamin D-gene-related methylation, we performed methylated DNA immunoprecipitation (MeDIP) using a commercially available kit (active Motif), following the manufacturer’s protocol (29). Briefly,
10 μg of genomic DNA was sheared four times at 30% amplitude to generate DNA fragments of 200–500 bp. 5-Methylcytosine was then immunoprecipitated overnight with a specific antibody, washed, eluted, and purified before real-time SYBR green PCR. The following primers were used: ovine 24a1 forward 5’-TCACGTCGGCTCTTCCTCTCTCCTTCT-3’, reverse 5’-GGGCTCTTGATACAGCCAGA-3’; bovine 27b1 forward 5’-CCAGGAAAAGTCGGAAGA-3’, reverse 5’-TCCTCAGCAATCTCTCTC-3’; and bovine VDR forward 5’-GGATTATGCGAGCAACAC-3’, reverse 5’-ATCAACCCAGCG-CACTCTCTC-3’. These primer sets amplified a short segment of the proximal promoters of CYP24A1 (~297 to ~78 bp of translation start site; 19 CpGs), CYP2B7b1 (~250 to ~101 bp of translation start site; 5 CpGs), and VDR (~303 to ~156 bp of transcription start site of the most common mRNA variant and ~25,584 to ~25,437 bp of translation start site; 9 CpGs).

Statistical analysis. All data are presented as means ± SE. We used univariate analysis to determine the differences for each variable according to the two independent variables (oxygen level and developmental stage) using SPSS 16.0 software (IBM, Armonk, NY). Equal variances were confirmed by Levene’s test. Statistical significance was determined as P < 0.05.

RESULTS

Effect of pregnancy and high-altitude long-term hypoxia on vitamin D status. Previous studies on this ovine model have shown that normoxic and hypoxic pregnancies have similar outcomes but altered physiological adaptations (11, 31, 39). To assess the vitamin D status in this model, we analyzed the plasma levels of vitamin D metabolites [cholecalciferol, 25-OH-D, and 1α,25-(OH)2D] in nonpregnant and pregnant ewes, and their fetuses at normoxia (NMX; sea level) and long-term hypoxia (LTH; 3,800 m above sea level). There were no differences in the levels of cholecalciferol between nonpregnant and pregnant ewes; however, fetal sheep had significantly higher levels of 25-OH-D than the adult sheep in both NMX and LTH groups (Fig. 1A). The levels of 25-OH-D were <40 nM in all NMX groups (nonpregnant, pregnant, and fetal sheep), and LTH-exposed sheep have more than twice the levels of 25-OH-D than NMX sheep at all three developmental stages (nonpregnancy, pregnancy, and fetal) (Fig. 1B). Therefore, NMX sheep are vitamin D insufficient and LTH sheep are vitamin D deficient as defined by the human standard levels of 25-OH-D. In contrast, the levels of the bioactive metabolite 1α, 25-(OH)2D in NMX and LTH ewes are within the reported range of vitamin D sufficiency in humans. Furthermore, similarly to humans, maternal plasma levels of 1α,25-(OH)2D were increased significantly in pregnancy compared with nonpregnancy (Fig. 1C). Interestingly, LTH further increased the circulating levels of 25-hydroxyvitamin D in all three groups (nonpregnancy, pregnancy, and fetal; Fig. 1C). The ratio of bioactive vitamin D in pregnant to nonpregnant sheep was 2.5 for NMX and 2.2 for LTH sheep. Fetuses had significantly higher levels of bioactive vitamin D compared with pregnant and nonpregnant adult sheep, with ratios of fetus-to-pregnant adult of 7.1 for NMX and 3.6 for LTH (Fig. 1B). These data indicate that the developmental stage (fetus, nonpregnant, and pregnant adult) has great significance in the regulation of the plasma levels of bioactive vitamin D without affecting the levels of precursors such as cholecalciferol and 25-OH-D. Our second independent variable (hypoxia) increased vitamin D status by increasing the levels of 25-OH-D and bioactive vitamin D. The LTH-to-NMX ratios were similar between developmental stages for both 25-OH-D (2.2 for nonpregnant, 2.3 for pregnant, and 2.6 for fetal) and 1α,25-(OH)2D (2.7 for nonpregnant and 2.4 for pregnant) metabolites (Fig. 1, B and C). Only the LTH-to-NMX ratio of 1α,25-(OH)2D in fetuses was not altered. Therefore, chronic hypobaric hypoxia has an independent effect on vitamin D status in this mammalian species.

Although the levels of bioactive vitamin D were increased significantly by both pregnancy and LTH, the total calcium plasma levels were not significantly different between nonpregnant and pregnant or NMX and LTH groups, with the exception of higher total calcium levels in fetuses compared with ewes (Fig. 1D). Similarly, the circulating levels of the VDBP were similar between NMX and LTH ewes and similar between pregnant and nonpregnant ewes. However, plasma VDBP was significantly lower in fetuses compared with adult ewes, and again LTH did not have a significant role (Fig. 1E). Finally, we analyzed the plasma levels of iPTH, as it correlates with expression of CYP27b1 and the production of bioactive vitamin D (8, 33). We found that iPTH was similar between pregnant and nonpregnant ewes, but LTH significantly increased the iPTH levels in both nonpregnant and pregnant ewes. Fetuses of both NMX and LTH pregnancies had significantly higher iPTH levels than their corresponding ewes (Fig. 1F). Therefore, iPTH levels correlated with increases in bio-

Fig. 2. Effect of pregnancy and hypoxia on maternal expression of 25-hydroxylases. Renal cortex (A) and liver (B) from pregnant (P) and nonpregnant (NP) ewes were processed to analyze mRNA expression of 25-hydroxylases CYP27A1, CYP2J, CYP27A1, and CYP3A4 by real-time PCR, as described in MATERIALS AND METHODS. Results are shown as averages ± SE (n = 4–5/group). *P < 0.05, LTH vs. NMX; #P < 0.05 NP vs. P.
active vitamin D and total calcium levels observed in fetuses of both NMX and LTH pregnancies.

**Pregnancy and LTH effects on maternal expression of vitamin D-relevant genes.** First, we investigated the effect of LTH and pregnancy on 25-hydroxylases CYP2R1, CYP2J, CYP27A1, and CYP3A4 on the ewe’s liver and kidneys. As shown in Fig. 2A, LTH upregulated the renal expression of CYP2R1 and CYP2J significantly by approximately twofold in both nonpregnant and pregnant ewes. There were no differences in the expression of CYP27A1 or CYP3A4. In contrast, the expression of 25-hydroxylases in the liver was significantly different compared with that of the kidney (Fig. 2, A and B). LTH significantly decreased the expression of CYP27A1, CYP3A4, and CYP2J in nonpregnant and pregnant ewes. Furthermore, pregnant ewes had significantly higher expression of CYP27A1 and CYP2J but lower expression of CYP3A4 compared with nonpregnant ewes in both the NMX and LTH groups (Fig. 2B). Therefore, both pregnancy and chronic hypoxia significantly altered the expression of 25-hydroxylases in the liver.

We then analyzed the renal expression of the two CYP450 enzymes involved in the activation (CYP27b1) and inactivation (CYP24a1) of bioactive vitamin D. We found that both pregnancy and high-altitude hypoxia significantly upregulated the levels of CYP27b1 mRNA and protein (Fig. 3, A and B), and the interaction between the two factors (LTH and pregnancy) was significant (P = 0.002 for mRNA levels and 0.05 for protein levels). In contrast, pregnancy did not induce any significant changes in CYP24a1 mRNA/protein levels, and only LTH significantly decreased CYP24a1 protein (but not mRNA) levels compared with NMX controls (Fig. 3, C and D). Because of LTH downregulation of CYP24a1, the ratios of CYP27b1/CYP24a1 protein expression were higher in LTH vs. NMX kidneys (1.06 in pregnant LTH vs. 0.53 in pregnant NMX). In addition, we analyzed renal expression of vitamin D transport genes such as VDBP, megalin, and cubulin and found no significant differences between our four groups of ewes (data not shown). Therefore, in this ovine model of mammalian pregnancy, increased levels of bioactive vitamin D correlate with maternal renal upregulation of CYP27b1 without alteration of CYP24a1 expression.

Several studies have established a default negative feedback mechanism of vitamin D status that involves VDR-mediated upregulation of CYP24a1 (5, 21). Because we did not observe CYP24a1 upregulation in either pregnancy or LTH in the presence of higher vitamin D status, we investigated the renal expression of VDR. The levels of VDR mRNA were not significantly different between the groups, but the levels of VDR protein were significantly lower in pregnant compared with nonpregnant groups and between LTH compared with NMX groups (Fig. 4B). However, the interaction between pregnancy and LTH factors was not
significant ($P = 0.11$), indicating there was no synergism between the two independent variables. The ratio of VDR to CYP24a1 protein expression in pregnancy vs. nonpregnancy decreased significantly in both the NMX and LTH groups (0.48 and 0.33 for nonpregnant NMX and LTH vs. 0.22 and 0.2 for pregnant NMX and LTH, respectively). This indicates that pregnant kidneys of both NMX and LTH ewes have lower VDR molecules available for activation by ligand and downstream regulation of target genes such as CYP24a1, thereby limiting autoregulation via VDR in maternal kidney. To determine whether pregnancy and LTH altered VDR expression in other VDR-expressing tissues, we analyzed the expression of VDR protein in duodenum, peripheral mononuclear cells (PBMC), and lung tissue in our four ewe groups. We found no significant differences in the levels of VDR protein in either PBMC or duodenum (Fig. 4, C and D). The pulmonary nuclear VDR levels were decreased by pregnancy and LTH, and the interaction of the two independent factors was significant (Fig. 4E). Therefore, VDR downregulation was a pregnancy-driven event that is tissue specific.

Role of LTH on placental and fetal expression of vitamin D-relevant genes. Fetal and placental weights were not significantly different between normoxic and hypoxic pregnancies (9.1 ± 0.7 and 0.75 ± 0.11 lbs. for normoxic fetal and placental weights, respectively; 8.9 ± 0.6 and 0.77 ± 0.11 lbs. for hypoxic fetal and placental weights, respectively). Recent reports have shown that LTH alters endocrine, vascular, and pulmonary physiology in the fetus (11, 31, 39). For this reason, we studied the effect of LTH on placental and fetal expression of vitamin D metabolic, transport, and receptor genes. We found that LTH placentas...
had significantly higher expression of CYP27b1 mRNA and protein, with LTH/NMX ratios of 1.9 (mRNA) and 2.1 (protein) (Fig. 5, A and B). In contrast, LTH placentas showed significant downregulation of CYP24a1 mRNA and protein compared with NMX placentas, with ratios of 0.5 (mRNA) and 0.37 (protein) (Fig. 5, A and B). Of interest is that although VDR protein levels were significantly lower in LTH compared with NMX in renal tissue, the opposite was true for placental tissue, with LTH/NMX ratios of 2.1 (Fig. 5B). LTH also upregulated the placental expression of VDBP but had no effect on vitamin D transporters megalin and cubulin (Fig. 5C). LTH did not significantly alter the expression of CYP27b1 and CYP24a1 in fetal kidney cortex (data not shown). Finally, LTH significantly downregulated VDR expression in fetal lung (Fig. 5D), with no effects on fetal kidney cortex (Fig. 5D), umbilical cord PBMCs, or fetal duodenum (data not shown). Our data suggest that LTH alters placental and fetal gene expression that further increases vitamin D status.

Role of pregnancy and LTH on vitamin D-related gene promoter methylation. Previous studies have suggested a potential role for promoter methylation, particularly placental CYP24a1 (35), on vitamin D status during pregnancy. To explore the possibility of promoter methylation on the regulation of CYP24a1, CYP27b1, and VDR, we performed methyl DNA immunoprecipitation (MeDIP) assays in pregnant and nonpregnant ewe kidney cortices and corresponding placentas (n = 3 for each group and tissue). We found that CYP24a1 had methylation levels of ~2%, CYP27b1 had low methylation levels of <0.5%, and VDR promoter had no methylation in placental or kidney tissues of either NMX or LTH samples (data not shown). Therefore, in this model, the levels of VDR and CYP24a1 proximal promoter methylation are very low and suggest that gene silencing via methylation is not a major mechanism in the regulation of these genes by LTH and pregnancy.

DISCUSSION

The first objective of this study was to characterize the pregnancy-specific regulation of vitamin D status and metabolism in an ovine model. We found that the sheep species is a model of healthy pregnancy characterized by increased maternal circulating levels of bioactive vitamin D without symptoms of vitamin D toxicity (hypercalcemia). Therefore, the pregnancy-specific regulation of vitamin D status in the sheep is similar to that reported in humans (9, 40) and rats (12). Increased vitamin D status in the pregnant ewe is associated with a disruption of the negative feedback mechanism that involves VDR-mediated upregulation of CYP24a1. This well-characterized mechanism in nonpregnant adult mammals is important in decreasing higher-than-normal circulating levels of the bioactive molecule 1α,25-(OH)2D (8, 33). We have now shown in both rats and sheep that increases in vitamin D status during pregnancy are due to vitamin D metabolic gene expression changes in maternal kidney and placenta. We propose the following pregnancy-specific mechanisms that lead to increased vitamin D status: 1) renal and placental upregulation of
the vitamin D-activating enzyme CYP27b1 together with 2) disruption of VDR-mediated upregulation of the vitamin D-inactivating enzyme CYP24a1 (Table 2). Part 2 is achieved partly by decreasing the renal expression of VDR, thereby limiting VDR-dependent upregulation of CYP24a1. Therefore, whereas CYP27b1 upregulation leads to increased production of 1α,25-(OH)2D, VDR and CYP24a1 levels do not concomitantly increase, thereby allowing a prolonged increase in circulating levels of bioactive vitamin D. This increase in vitamin D status, however, does not lead to hypercalcemia or cause other vitamin D-related toxicity but has been proposed to ensure healthy pregnancy outcomes (4, 23, 24, 46). In contrast to pregnancy, aging has been associated with a different disruption of vitamin D metabolism, in which lower levels of bioactive vitamin D are associated with increased expression of renal CYP24a1 (3). Future research is needed to uncover the pregnancy-specific factors that regulate CYP27b1, CYP24a1, and VDR, the maternal adaptations to prevent vitamin D toxicity, and the noncalcemic roles of vitamin D during pregnancy, including its role in placental and fetal development.

Our second objective was to investigate the effect of high-altitude chronic hypoxia (LTH) on vitamin D status in adult nonpregnant and pregnant ewes and their fetal lambs. Both hypoxia and vitamin D deficiency are considered risk factors for pregnancy complications and increased maternal/fetal mortality and morbidity (6, 16, 24, 30, 52, 53); however, little is known about the effects of chronic hypoxia, as occurs in the high-altitude lifestyle, on vitamin D status and metabolism. We found that LTH exposure was associated with further increases in vitamin D status [shown by higher circulating levels of both 25-OH-D and 1α,25-(OH)2D levels] in ewes and their fetuses. The effects on the stable precursor and bioactive vitamin D metabolites were not the result of increased ultraviolet B radiation exposure, as the levels of cholecalciferol in LTH ewes were similar to those in NMX ewes. Increased maternal plasma levels of 25-OH-D correlated with upregulation of renal 25-hydroxylases CYP2J1 and CYP2R1 (Table 2). LTH-exposed ewes, however, had significant liver downregulation of 25-hydroxylases that could be a feedback mechanism to increased 25-OH-D levels or an independent LTH response in this tissue. LTH also significantly increased the levels of bioactive vitamin D that could be a result of increased levels of 25-OH-D in addition to renal upregulation of CYP27b1. Of interest, LTH increased the plasma levels of PTH that are known to stimulate the renal expression of CYP27b1 by transcriptional activation through cAMP signaling (33). PTH was also significantly higher in fetuses compared with their matching ewes, and this event correlates with four- to seven-fold higher levels of bioactive vitamin D in umbilical cord compared with maternal circulation. Therefore, our data suggest that LTH increased ewe and fetal PTH-dependent upregulation of CYP27b1 that is shown at the mRNA and protein levels in both maternal renal cortex and placental tissues (Table 2). The effect of LTH on PTH levels explains the synergistic interaction of LTH with pregnancy on increased levels of bioactive vitamin D. Finally, our study revealed that this ovine LTH model is unique in showing LTH decreases in renal CYP24a1. Previous in vitro studies have shown that hypoxia upregulates the expression of CYP27b1, CYP24a1, and VDBP gene expression in human cells (1, 27). The effect of hypobaric hypoxia on VDR-related gene expression is thought to be indirect, as there are no consensus hypoxia-inducible factor-1α DNA-binding elements in these genes (8, 19). Our studies suggest that LTH effects on CYP24a1 and VDR were mediated at the protein level since mRNA levels were not altered. In general, hypoxia is well known to selectively decrease protein translation (49), and therefore, we hypothesize that LTH decreased renal expression of VDR and CYP24a1 via decreased protein translation, a hypothesis that warrants further study. To our knowledge, this is the first report on the effects of LTH in vitamin D status during mammalian pregnancy. In humans, deficient levels of maternal 25-OH-D have been adjudicated to cultural lifestyle habits that lead to decreased sun exposure, but the effect of high-altitude hypoxia on vitamin D status in nonpregnant or pregnant mammals has not been addressed. Studies on the effect of gestational hypoxia on rat pregnancy outcomes indicated that hypobaric hypoxia of 5,000 m but not of 3,000 m resulted in IUGR; however, disruption of nursing and maternal and offspring HPA axis was observed at both altitude levels (7). Our ovine model has shown HPA axis alterations in association with normal fetal and placental weights similar to this rat model (31). It would be of great interest to study the vitamin D metabolism in this rat model of gestational hypoxia to elucidate whether regulation similar to our ovine model occurs. Finally, our study revealed that LTH caused significant downregulation of both fetal and maternal lung VDR, possibly altering downstream effects of vitamin D on this nonclassical target organ, and therefore, hypoxia-dependent VDR downregulation could be an important molecular event in the pathogenesis of hypoxia-driven pulmonary disease (39). Altogether, our studies on LTH effects on ovine pregnancy led us to hypothesize that increased vitamin D status could be an adaptive mechanism to ensure adequate placental and fetal growth under hypoxic conditions. Uncovering the molecular mechanisms of this adaptation to LTH might lead us.

Table 2. Effect of pregnancy and high-altitude LTH on vitamin D status and metabolism

<table>
<thead>
<tr>
<th>Maternal vitamin D status</th>
<th>Effect of Pregnancy</th>
<th>Effect of LTH†</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH-D</td>
<td>No difference*</td>
<td>Increased</td>
</tr>
<tr>
<td>1α,25-(OH)2D</td>
<td>Increased*</td>
<td>Increased</td>
</tr>
<tr>
<td>Total Ca</td>
<td>No difference*</td>
<td>No difference</td>
</tr>
<tr>
<td>VDBP</td>
<td>No difference*</td>
<td>No difference</td>
</tr>
<tr>
<td>iPTH</td>
<td>No difference*</td>
<td>Increased</td>
</tr>
<tr>
<td>Maternal liver 25-hydroxylases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP27a1</td>
<td>Increased*</td>
<td>Decreased</td>
</tr>
<tr>
<td>CYP2J</td>
<td>Increased*</td>
<td>Decreased</td>
</tr>
<tr>
<td>CYP3a4</td>
<td>Decreased*</td>
<td>Decreased</td>
</tr>
<tr>
<td>Maternal kidney vitamin D metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-Hydroxylases</td>
<td>No difference*</td>
<td>Increased</td>
</tr>
<tr>
<td>CYP27b1</td>
<td>Increased*</td>
<td>Increased</td>
</tr>
<tr>
<td>CYP24a1/VDR</td>
<td>No difference/decreased*</td>
<td>Decreased/decreased</td>
</tr>
<tr>
<td>Fetal vitamin D status 25-OH-D</td>
<td>No difference‡</td>
<td>Increased‡</td>
</tr>
<tr>
<td>1α,25-(OH)2D</td>
<td>Increase‡</td>
<td>Increased‡</td>
</tr>
<tr>
<td>Total Ca</td>
<td>Increased‡</td>
<td>No difference</td>
</tr>
<tr>
<td>VDBP</td>
<td>Decreased‡</td>
<td>No difference</td>
</tr>
<tr>
<td>iPTH</td>
<td>Increased‡</td>
<td>No difference</td>
</tr>
<tr>
<td>Placental vitamin D metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP27b1</td>
<td>Increased§</td>
<td>Increased</td>
</tr>
<tr>
<td>CYP24a1/VDR (uncoupled)</td>
<td>No difference/increased§</td>
<td>Decreased/increased</td>
</tr>
</tbody>
</table>

LTH, long-term hypoxia. *Compared with nonpregnant ewe; †compared with normoxic; ‡compared with maternal; §compared with fetal kidney.
into finding new molecular targets and treatments to improve vitamin D status in humans exposed to chronic hypoxia.

Finally, our study revealed that VDR expression regulation by pregnancy and hypoxia is tissue dependent. VDR expression was significantly decreased in maternal renal tissue but increased in placental tissues at term, with VDR/CYP24a1 ratios of 0.87 vs. 0.22 in NMX placenta vs. maternal kidney and 5.0 vs. 0.2 in LTH placenta vs. maternal kidneys. LTH did not induce downregulation of VDR expression in placenta as it did in maternal kidney and lung, again showing tissue-dependent regulation. Our studies also suggest that fetal vitamin D status is regulated mostly by the placenta because LTH had a higher impact on placental vitamin D metabolic gene expression compared with fetal kidney (Table 2). Therefore, the VDR/CYP24a1 negative feedback axis is further disrupted in placental tissue compared with maternal kidney tissue. Our studies on promoter methylation revealed that this mammalian species has significantly lower CYP24a1 promoter methylation compared with human and mouse species (35) and similar to other species reported, such as in the cow and the cat (32). Therefore, further research is required to reveal the mechanisms by which placental expression of CYP24a1 is blunted throughout pregnancy in the presence of high levels of VDR and its natural bioactive ligand 1α,25(OH)2D. Some possible mechanisms of placental-specific CYP24a1 downregulation include microRNA, VDR/corepressor, and enhancer/intron methylation silencing (8, 14, 32). It is important to note that the gene expression changes in kidney and placenta correlated with changes in vitamin D status, in contrast with gene expression observed in other tissues such as liver, duodenum, and PBMCs. Our studies indicate that the kidney and placenta are the main tissues that regulate vitamin D homeostasis during pregnancy.

In summary, this study further confirms that pregnancy disrupts the negative feedback mechanism of VDR-mediated upregulation of CYP24a1, thereby allowing an increase in bioactive vitamin D levels. Decreased CYP24a1 protein levels are associated with lower levels of VDR expression in maternal renal cortex. Increased levels of VDR expression in placental tissues suggest an important role of VDR in placental development. LTH alone increased vitamin D status by upregulation of 25-hydroxylase expression, upregulation of CYP27b1, and further downregulation of CYP24a1 in both placental and maternal renal tissues. We conclude that our ovine model of pregnancy has similar physiological and molecular adaptations that lead to increased vitamin D status as those that occur in other mammal species such as rodents and humans. Furthermore, this mammalian species responded to LTH with further increases in vitamin D status in association with vitamin D metabolic gene expression regulation. We hypothesize that some humans residing at high altitude could have similar physiological adaptations to chronic hypoxia that lead to improved endogenous bioactivation of vitamin D. Future research is needed to discover the mechanisms that lead to LTH increases in vitamin D status and confirm whether this LTH-specific event is involved in preventing certain pregnancy complications like IUGR and preeclampsia.

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REFERENCES


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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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